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Review

Intact protein bioanalysis by liquid chromatography – High-resolution mass spectrometry

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1. Introduction

Liquid chromatography coupled online to mass spectrometry (LC-MS) is arguably the most widely used bioanalytical technique due to its sensitivity and selectivity. It is a very versatile analytical approach that can be tuned to address a wide range of molecules with applications from environmental analysis to drug analysis and doping control. LC-MS is increasingly used for the analysis of proteins, typically after a proteolytic digestion step which converts the macromolecular analytes into lower-molecular weight peptides, which can be readily quantified. LC-MS analysis of intact proteins, however, is still in its infancy, being mainly restricted to the detailed characterization of biopharmaceuticals in dose formulations [1–10]. The analysis of intact proteins in complex biological samples, referred to here as protein bioanalysis, has remained the realm of ligand binding assays (LBAs) and notably of enzyme-linked immunosorbent assays (ELISAs) due to their exquisite sensitivity and specificity. A disadvantage of ELISAs is that specificity can often not be assessed due to a lack of alternative analytical techniques and the fact that the binding site is not known (or not provided by the manufacturer). Despite considerable advances in mass spectrometry and liquid chromatography, protein bioanalysis by LC-MS remains challenging due to the fact that a given (set of) protein(s) needs to be detected in a sensitive and selective manner in a matrix of (hundreds of) thousands of other proteins. The task is further complicated by the fact that proteins are not very amenable to LC separation and that they generate complex mass spectra upon electrospray ionization (ESI), the most widely used ionization method for protein analysis by LC-MS, due to multiple charge states (so-called charge-state envelopes). Quantitative protein bioanalysis, notably in the area of biopharmaceuticals, would, however, benefit from analysis at the intact protein rather than at the peptide level, since protein species (also known as proteoforms) [11–18] should be separated from each other to facilitate the assignment of modifications to a given species. Despite these incentives, it is fair to say that protein bioanalysis is currently dominated by the bottom-up approach using so-called signature or proteotypic peptides as surrogates for the proteins of interest, since LC-MS analysis at the protein level is often not sensitive and specific enough.

This review discusses the challenges of quantitative protein bioanalysis by LC-MS at the protein level. We will notably address the possibilities and current limitations of protein sample preparation, separation by LC, the challenge of interpreting protein ESI-MS spectra and the options for protein quantification based on extracted ion chromatograms or deconvoluted spectra. The possibilities of high-resolution mass spectrometry (HRMS) with respect to improving the signal-to-noise (S/N) ratio and the challenges of analyzing complex mass spectra will be highlighted based on examples.

2. Sample preparation: a critical step in protein bioanalysis

The bioanalysis, and notably the quantification, of therapeutic proteins (biopharmaceuticals) and macromolecular biomarkers in complex biological samples like serum or plasma requires dedicated sample preparation. Because of the often low concentrations of the protein analyte and the presence of many endogenous proteins that easily interfere in the LC-MS analysis, it is essential that the sample should be cleaned up and the protein(s) of interest enriched [19].

Enrichment strategies for intact proteins are typically based on selectively capturing the protein of interest with an affinity ligand such as an antibody, receptor or another affinity binder. More generic enrichment techniques, such as solid-phase extraction (SPE) using electrostatic or hydrophobic interactions, immobilized metal affinity columns (IMAC) or fractionated protein precipitation can also be applied but they require careful optimization based on the physical-chemical properties of the target protein to obtain sufficient selectivity [20].

Sample preparation for intact protein analysis by LC-MS, and specifically affinity-based enrichment, depends on the specificity of the affinity ligand. Monoclonal-, polyclonal- or engineered recombinant antibodies as well as biological receptors are widely used as capturing agents. For each type of ligand, it is important to know against which part of the protein analyte it is directed and which proteoform of the protein is captured, to allow proper interpretation of the results. For

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example, when using the biological receptor to capture a protein, it must be considered that only protein species that retain affinity for the receptor will be enriched, indicating that they still have at least some biological activity. Any proteoform of the protein that has undergone enzymatic (e.g. by proteolysis) or chemical modifications (e.g. by oxidation or deamidation) at the site of binding and, as a consequence, has lost its ability to bind to the receptor, will not be captured and thus escape analysis.

There is a growing number of affinity binders that are based on other protein scaffolds. Examples are DARPins, aptamers and affimers. DARPins (Designed Ankyrin Repeat Proteins) are genetically engineered proteins typically exhibiting highly specific and high-affinity target protein binding [21]. They are derived from natural ankyrin proteins, one of the most common classes of binding proteins in nature. Affimers are affinity binders based on a structurally robust protease inhibitor scaffold (e.g. Cystatin A) [22,23]. Both types of binders are selected by phage display against the target protein and produced in an *E. coli* protein expression system. DARPins and affimers have a defined amino acid sequence with defined binding regions that are amenable to protein engineering (e.g. the insertion of a unique cysteine residue for immobilization in affimers). Multiple affimers may be used in a single assay to capture several proteins and to analyze them by LC-MS [24].

If a broad recognition of the different forms of a protein analyte is desired, it is sometimes advantageous to use polyclonal antibodies that recognize multiple epitopes. However, this may come at the price of more unspecific binding. As often is the case with complex analytical problems, there is no 'one size fits all' solution and complementary strategies may have to be used to get a comprehensive view of the species that are related to a given protein. An important parameter to be optimized during method development is the ratio of the carrier (e.g. the magnetic bead volume) to the amount of capturing affinity binder, which must be related to the estimated maximum protein concentration, to determine at which point all binding sites are saturated. Such 'titration experiments' prevent inaccurately low results at high analyte concentrations due to antibody saturation and competition between protein species for binding. In general, for all types of sample preparation approaches, it is critical to assess and ideally avoid non-specific binding of proteins to carrier materials to arrive at reproducible results with good recovery.

Affinity ligands can be coupled to polymer resins, magnetic beads, immobilized on 96-well enzyme-linked immunosorbent assay (ELISA) microtiter plates or on monolithic micro-columns. The coupling chemistry depends on the reactive groups on the carrier and the functional groups on the affinity ligands. It is beyond the scope of this review to cover this field completely, but a few examples will be described to delineate the principle. An antibody can, for example, be biotinylated using an N-hydroxysuccinimide biotin derivative that reacts with primary amino groups on lysine residues and the N-termini of the heavy and light chains. The biotinylated antibody can then be coupled to a streptavidin-labelled carrier, because of the strong interaction between biotin and streptavidin. When using an ELISA plate, there is a choice between unmodified and a variety of surface-activated microtiter plates. As in the case of setting up a regular sandwich ELISA assay, it is necessary to evaluate different types of ELISA plates for the best recovery and selectivity. There are many examples where affinity-based sample preparation is used for protein analysis, although enrichment is typically followed by trypsin digestion and LC-MS analysis at the peptide level [25]. Kellie et al. described immunocapture to enrich a monoclonal antibody (mAb) from human plasma followed by digestion with IdeS (see later in this section for details about this approach) and reduction of the disulfide bonds [26]. LC-MS analysis on a C_4 reversed-phase column at 65 °C allowed separation of the Fc/2, LC and Fd subunits. The LC subunit of the captured antibody was used as internal standard to correct for losses. The method was quantitative across the range of 0.5–10 µg/mL based on Extracted Ion Chromatograms (EICs) of the 20^+ charge state. Other examples of intact protein bioanalysis are scarce, but many of the 'peptide-based' methods might serve as the basis for intact protein analysis by analyzing the proteins that are eluted from the capturing agents directly by LC-MS without prior digestion. Immunoaffinity-MS analysis of intact proteins has also been shown successfully in conjunction with matrix-assisted laser desorption ionization (MALDI) albeit for relatively small proteins [27–29].

Since protein A and protein G show affinity towards the constant part of human IgG, carriers can be coated with one or a combination of both proteins in order to immobilize capturing antibodies or to extract therapeutic mAbs. There are positive and negative aspects of this kind of sample preparation. Advantageous is that such methods can be applied to different types of proteins containing IgG-like Fc parts. They are robust, can be used to capture multiple therapeutic antibodies (or their protein species) in one analysis and are easy to transfer to 96-well plate formats. A negative aspect of using protein A and/or G is that they bind all human IgG molecules, of which there are many in blood serum or plasma, at a total concentration of 15 mg/mL, so that analysis at the protein level is generally not possible. In recent years, affinity enrichment has been shifting from manual sample preparation to automated platforms based on well-plate-, magnetic bead-, cartridge- or tip-based immunocapture platforms. Automation leads to improved precision, shorter sample preparation times and more robust methods, because samples can be processed (captured, washed and eluted) according to the same protocol and often simultaneously.

The affinity enrichment method described by Berna et al. [30] incorporates a sample preparation step using immunoprecipitation (IP) for quantitative protein analysis by LC-MS. IP was set up in a 96-well plate format using protein A/G to immobilize the capture antibody. The authors refer to this technique as immunoprecipitation in ELISA format or IPE. Proteins were eluted from the antibodies with 5% aqueous acetic acid, which is MS compatible, so this method could also be used for intact protein analysis. This approach, with some modifications, was applied by Klont et al. [31], to enrich the soluble Receptor of Advanced Glycation End-products (sRAGE), a biomarker that is under investigation in Chronic Obstructive Pulmonary Disease (COPD), from serum and to quantify it by LC-MS at the sub-ng/mL level after digestion.

Reversed-phase SPE is commonly used for the enrichment of proteins between 10 and 35 kDa. With relatively small pore sizes of 80–130 Å (8–13 nm), the separation is not only based on hydrophobic interactions of the proteins with the stationary phase, but also on size-exclusion effects, which helps remove larger, highly abundant proteins like albumin or IgGs from serum or plasma, as they cannot penetrate into the pores. An example of reversed phase SPE for protein enrichment is the quantification of human growth hormone (approx. 22 kDa) in serum by Pritchard et al. [32]. These authors used a two-step reversed phase SPE process at high and low pH for enrichment at the intact protein level prior to tryptic digestion directly on the C_{18}-based SPE material. Monolithic SPE phases may be beneficial for protein extraction for several reasons. Monolithic column materials consist of a single piece of a highly porous structure that is silica- or polymer-based [33,34]. Due to the high porosity of the material and the large accessible surface area, binding of high-molecular-weight proteins is improved. Yang et al. [35] used monolithic C_{18} SPE for quantification of PEGylated interferon alpha-2a (approx. 40 kDa) at the low ng/mL level in serum. These levels were achieved with LC-MS analysis at the peptide level, while the monolithic SPE step was performed at the protein level.

The use of IMAC-SPE during sample preparation is rather uncommon for protein enrichment, whereas it is widely used for the enrichment of phosphorylated peptides. Some proteins have a particularly high affinity for metal ions, notably for doubly or triply charged transition metal cations. Surface-exposed histidine residues are known to exhibit affinity for immobilized Ni^{2+}, a feature that was used by Wilffert et al. [20] for the enrichment of recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL) from serum. In analogy to the enrichment of phosphorylated peptides, IMAC is also used for the enrichment of proteins containing phosphorylated threonine, serine or

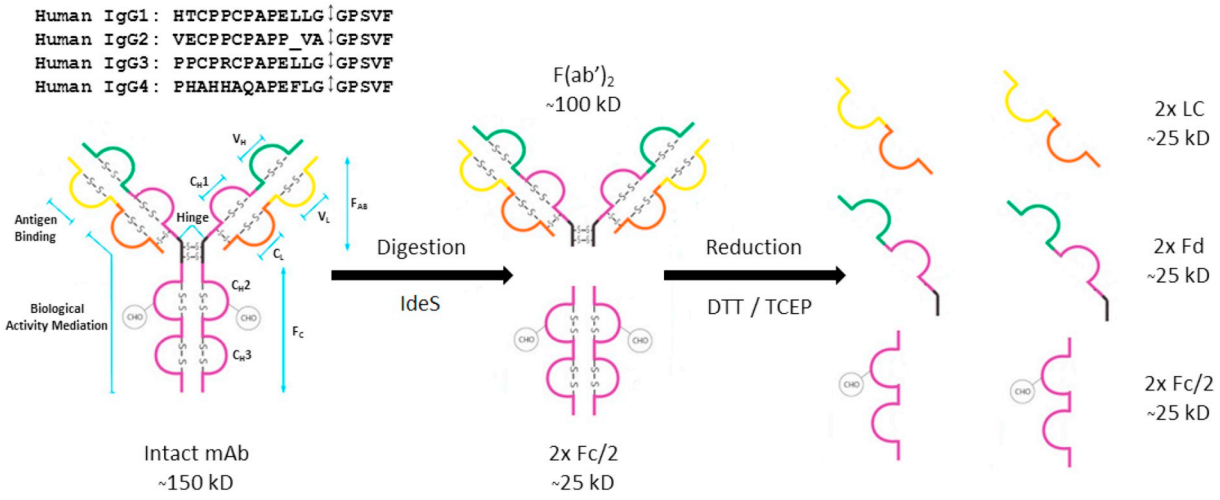

Fig. 1. Schematic representation of the digestion of an antibody (IgG) with IdeS followed by reduction of disulfide bonds resulting in Fd′, LC (light chain) and Fc/2 fragments of approximately 25 kDa each.

tyrosine residues, which have a strong affinity for trivalent metal ions like Fe^{3+}, Ga^{3+} and Al^{3+}.

When it comes to mAbs, the most widely used class of biopharmaceuticals, there is a specific approach for bioanalysis at the protein domain level after digestion with IdeS (immunoglobulin-degrading enzyme from *Streptococcus pyogenes*) [19–21]. IdeS is a cysteine protease that cleaves at the hinge region of all IgG subclasses. The enzyme is highly specific and no other substrate besides IgG is known [36,37]. Digestion of IgG with IdeS generates a homogenous pool of $F(ab')_2$ and Fc/2 fragments and there is no 'over-digestion' or further degradation of the fragments as is often the case with other proteolytic enzymes.

Since the hinge region of IgG molecules is flexible and solvent-exposed, digestion is fast. Digestion of IgG using IdeS produces two identical mAb subunits that correspond to the constant region of the heavy chain and are called Fc/2, plus a larger domain that is referred to as $F(ab')_2$. This consists of the two light chains (LC) and the variable parts of the heavy chains that are interconnected via a number of disulfide bonds. Treatment with a reducing agent cleaves the disulfide bonds and converts $F(ab')_2$ into two light chains and two separate variable parts of the heavy chain, Fd (Fig. 1). All subunits are approximately 25 kDa and thus more amenable to LC-MS analysis than the entire antibody, while still providing most of the molecular context for structural modifications.

The use of IdeS in the analysis and characterization of IgGs and fusion proteins at the protein domain level using liquid chromatography or capillary electrophoresis in combination with high-resolution mass spectrometry has been described in several reports, albeit primarily for pharmaceutical product control in samples of limited complexity [38–41]. An et al. [42] developed a method for the identification and routine monitoring of domain-specific modifications. Suitability of the methodology was demonstrated for a number of IgG subclasses (IgG1, IgG2 and IgG4), as well as for an Fc fusion protein. IdeS digestion was followed by reduction of disulfide bonds and subsequent analysis by LC-MS, capillary isoelectric focusing and glycan mapping to enable domain-specific profiling of oxidations, charge heterogeneity, and glycoform distribution. Leblanc et al. [43] used IdeS to study long-term storage stability of a proprietary mAb at 5 °C. An aged mAb, stored for nine years at 5 °C and presenting a complex charge variant profile, was investigated by cation-exchange (CEX)-LC and high-resolution MS. Mobile phases consisted of 50 mM ammonium formate buffered with formic acid at pH 3.9 (Buffer A) and 500 mM ammonium acetate, pH 7.4 (Buffer B), resulting in both a pH and a salt gradient. Peptide mapping was subsequently used to localize modified sites and provide quantitative information. Results showed a remarkable consistency of the data. IdeS digestion has also been implemented for the comparison of originator mAbs and biosimilars. Pisupati et al. [44] demonstrated the utility of a 'multiple-attribute monitoring' workflow using Remicade (Infliximab) and its biosimilar Remsima as models. IdeS digestion was used to determine the intact masses of the resulting fragments in their fully glycosylated state, after deglycosylation and/or disulfide bond reduction. They concluded that the workflow effectively proved that the two antibodies were similar, yet not identical. Reported differences, related to the levels of charge variants attributed to C-terminal truncation and dimer formation, were ultimately deemed non-consequential. Wagner-Rousset et al. [45] described the development of a rapid analytical platform to assess charge variants of mAbs (acidic and basic species). The workflow was based on comparative analysis by CEX of intact IgGs versus $F(ab)'_2$ and Fc domains generated by IdeS digestion. The analytical procedure was validated according to FDA and EMA guidelines in analogy to already approved mAb-based biopharmaceuticals. Functional assays and peptide mapping were performed to localize the modifications and assess their effect on biological activity. This approach can be used during the early stages of research and development of novel biopharmaceuticals to screen for and select optimized candidates by discriminating between critical and less critical charge variants, according to the CEX charge variant profiles of IdeS digested mAbs. This is an important feature since the identification of 'hot spots' is an important part in further (pre)-clinical development.

3. Protein separation by liquid chromatography

Liquid chromatography (LC) is one of the most powerful separation techniques and in combination with mass spectrometry is widely used in the field of bioanalysis. Due to their large size, intricate higher order structure and heterogeneity, chromatographic separation of proteins is extremely challenging. While sample preparation is a crucial and indispensable step to achieve sensitivity and selectivity in protein bioanalysis, it would not be possible to address protein heterogeneity to any appreciable extent without efficient LC separation prior to mass spectrometry. In the following, we will highlight some aspects of column technology as well as operational variables of chromatographic protein separations related to intact protein bioanalysis. Many aspects were developed for the characterization of therapeutic proteins and have only been partially translated to their analysis in complex samples. This is one of the main challenges lying ahead for protein bioanalysis.

Pioneering work of the groups of Regnier, Hearn and others in the 1980s established that efficient protein separation requires

chromatographic materials with large enough pores to allow access to the inner pore volume [46–53]. Insufficiently large pores lead to peak broadening due to slow mass transfer and may result in column fouling since not all proteins are eluted. A better theoretical understanding of protein separations by high-performance (HPLC) and later ultra-high-performance LC (UHPLC) led to the development of novel concepts, such as silica or polymer monoliths, with large, μm-sized flow-through pores and mesopores on the order of hundreds of nm allowing for efficient and rapid protein separation [54–59]. More recent developments in separation science based on the design and engineering of micropillar-containing columns instead of packed bed or monolithic materials hold promise that separation media for intact proteins can be designed based on fundamental chromatographic theory and nanoscale engineering [60–64]. While advances in protein separation by LC have been relatively slow over the last decades, we may be entering a new phase with breakthroughs ahead. As the following examples will show, such breakthroughs are sorely needed in order to advance LC separation of proteins to a level that is commensurate with the complexity of individual proteins such as biopharmaceuticals or biomarkers let alone with the complexity of entire proteomes. LC separation of intact proteins currently lags behind in separation power of two-dimensional gel electrophoresis, an approach that was developed in the 1970s, but which is cumbersome and not suitable for robust, quantitative protein bioanalysis.

Reversed-phase LC (RPLC) is one of the major analytical techniques used in the bioanalysis of proteins. RPLC as such is not ideal for protein analysis since the harsh mobile phase conditions and the elevated hydrophobicity of the stationary phase result in denaturation and may even induce protein precipitation or irreversible protein adsorption leading to poor recoveries and the loss of certain protein species. However, the wide range of stationary phases and notably the ease with which RPLC can be coupled to mass spectrometry counterbalance these disadvantages. Damen et al. [65] developed one of the first bioanalytical methods for the quantitative determination of intact trastuzumab, a monoclonal antibody for the treatment of metastatic breast cancer, by immunoaffinity enrichment followed by HPLC with fluorescence detection mainly based on Trp residues in the protein. Gradient elution was applied at a flow rate of 0.5 mL/min through a 150 × 2.1 mm ID column packed with a C_8 material with a particle size of 5μm and an average pore size of 300Å (30nm). The column temperature was 75 °C and mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) in water while mobile phase B consisted of 0.1% TFA in a mixture of isopropanol-acetonitrile-water (70:20:10). Coupling the LC separation to mass spectrometry resulted in insufficient sensitivity, indicating that efficient ionization of intact proteins is challenging. The authors suggested two reasons explaining the loss of sensitivity. Firstly, the high viscosity of the mobile phase as a result of the presence of isopropanol negatively affected spray performance. Secondly, the presence of TFA as ion pairing agent in the mobile phase led to ionization suppression. Thus, the authors decided to discontinue the use of mass spectrometry and switched to fluorescence detection. To prove the selectivity of the LC separation, trastuzumab was mixed with other therapeutic mAbs like adalimumab, bevacizumab, and rituximab. All components of the mixture were separated well. A number of limitations of this approach are obvious. First, fluorescence detection does not allow to gather chemical information from the analytes, such as molecular mass, or elucidate potential sources of heterogeneity. Second, while more sensitive than mass spectrometry, in this particular case, intrinsic Trp fluorescence is not very strong resulting in a limit of detection on the order of 5 μg/mL. While this is sufficient for highly dosed monoclonal antibodies like trastuzumab, it is insufficient for many other biopharmaceuticals and for most biomarkers. This is also due to the fact that almost every protein contains Trp, which results in a considerable background signal (Fig. 2). Mass spectrometry and notably high-resolution mass spectrometry allows more specific detection of proteins based on extracted ion chromatograms (EICs) with narrow mass

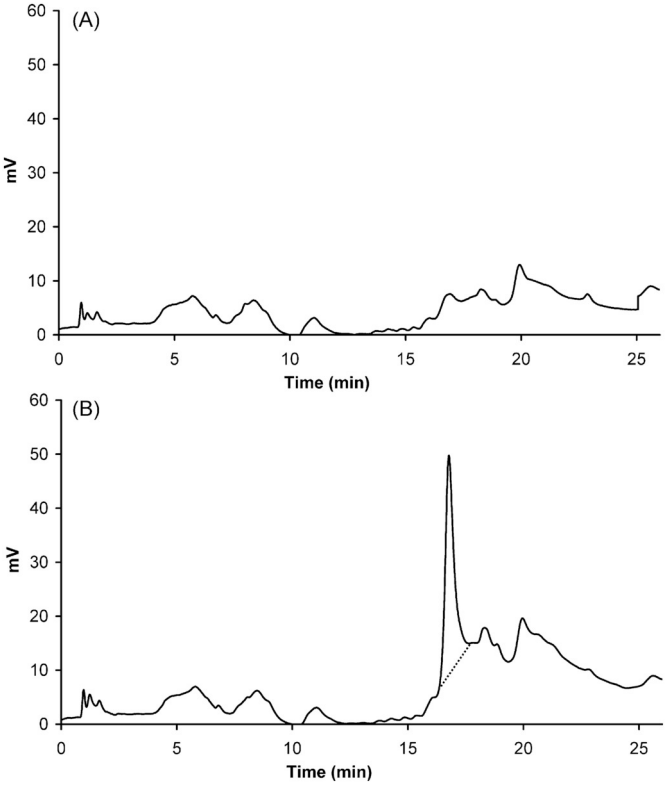

Fig. 2. Chromatograms of blank serum (A) and trastuzumab-spiked serum at the LLOQ (5 μg/mL) (B) after immunoaffinity enrichment using anti-trastuzumab idiotype antibodies. The retention time of trastuzumab is around 16.8 min (reproduced from Damen et al. 2009 with permission, copyright Elsevier).

extraction windows (MEWs) (see later in this article). However, the efficient ionization of proteins in their intact form remains a considerable challenge.

Another interesting feature of protein RPLC is that elevated temperatures lead to much improved separations. While this is a general phenomenon in LC, since the viscosity of the mobile phase decreases with increasing temperature, it is particularly striking for proteins. In their study of intact protein bioanalysis by high-resolution full scan mass spectrometry, Ruan et al. [66] used human lysozyme as a model protein to evaluate the effect of different column temperatures. While chromatography at room temperature resulted in a broad peak with poor peak shape, increasing the column temperature to 70 °C gave a narrow, symmetrical peak. The fact that different interchanging protein conformations may result in broad, asymmetric chromatographic peaks was already studied by Cohen et al. [67] in the 1980s, who reported that raising the column temperature is important for reducing the number of protein conformers (most likely by denaturing the protein into its unfolded state) and hence reducing peak broadening. Elevated column temperature is thus considered essential for efficient protein separation and notably for the separation and recovery of hydrophobic proteins and peptides. It can be concluded that sharp peaks at high temperatures are due not only to an increase in column efficiency but also due to reducing the number of conformational states of a given protein.

An acidic ion-pairing agent is typically added to the mobile phase of RPLC separations of peptides and proteins to improve chromatographic performance. TFA, at a concentration of 0.05–0.1% is widely used for this purpose since its introduction by Bennett et al. in 1977 [68] and further studies by Pearson et al. [69]. TFA reduces peak broadening and tailing since it binds to and neutralizes positively-charged amino acids thereby reducing interactions with silanol groups on the stationary

phase. TFA and its less widely used longer chain analogue heptafluorobutyric acid (HFBA), further improve solubility and strengthen the interaction with the hydrophobic stationary phase. While less effective in improving chromatographic separation efficiency, formic acid (FA) has gained in popularity for LC-MS separations of proteins and peptides, since TFA suppresses ion formation in the electrospray source. Jian et al. [70] established a workflow for the absolute quantitation of large therapeutic proteins in biological samples at the intact level by LC-HRMS. The authors used FA as a mobile phase additive rather than TFA, despite the fact that FA gave broader peaks, in favour of maximizing signal intensity. Somsen et al. [71] showed recently that the ionization suppression effect of TFA may be counterbalanced by adding a dopant gas to the electrospray interface. While this option is not available in all mass spectrometers, it is of interest to note that LC separation efficiency, which is better with TFA as ion pairing agent, may not have to be compromised in favour of a strong ESI-MS signal.

In the recent past, wide pore (> 300 Å) core-shell particles have been developed for the separation of proteins [72,73]. Core-shell particles, also called superficially porous particles, have a porous shell on top of a non-porous core resulting in a material with an efficiency that is comparable to particles of smaller diameter due to the shorter diffusion path length, resulting in fast mass transfer while producing less backpressure than UHPLC-type particles (1.7–1.8 µm diameter). Todoroki et al. [74] used a wide-pore core-shell RPLC column at 70 °C for the separation of purified mAbs as sharp peaks within 20 min. This approach was developed into a bioanalytical method for the quantification of bevacizumab and infliximab in human plasma by a combination of immunoaffinity enrichment, high-temperature RPLC and fluorescence detection.

Organic polymer-based monolithic columns emerged as an alternative to silica-based stationary phases for rapid and efficient biomolecule separation. A monolith is a cylindrically shaped polymer that contains a continuous and interconnected network of pores (channels). The first monolithic stationary phases were based on polymethacrylate or polystyrene-divinylbenzene in large inner diameter columns [75]. Nowadays, a wide variety of monomers is available for the preparation of polymer-based monoliths [75–77]. Polymer monoliths are stable at elevated column temperatures and across a broad pH range.

The porosity of polymers is a macromolecular characteristic that can be controlled by varying the composition of the polymerization mixture and the reaction conditions, such as polymerization temperature and time [77]. Most monolithic columns developed so far feature hydrophobic reversed-phase functionalities, because RPLC is generally regarded as the most effective separation mode. However, the wide variety of functional and cross-linking monomers allows the creation of monoliths carrying the desired surface chemistry to achieve LC separations in different modes. An example is an ion-exchange column based on a polymer monolith [78].

Monolithic materials are well-suited for the design of miniaturized columns [75]. The robustness of such columns can be increased via covalent linkage of the monolith to the inner capillary wall. Lanshoeft et al. used a 1 mm × 250 mm polymer-based monolithic column to develop an immunoaffinity enrichment, LC-HRMS workflow for quantification of a hIgG1 at the intact protein level in rat serum [79]. The column was based on an ethylvinylbenzene-divinylbenzene copolymer and the column temperature was raised to 70 °C for better separation and peak resolution.

Recently, Jin et al. reported a method for the quantitation of an intact antibody-drug conjugate (ADC), trastuzumab emtansine, in rat plasma [80]. Both trastuzumab and trastuzumab emtansine eluted at very similar (almost identical) retention times. The broader chromatographic peak for trastuzumab emtansine as compared to trastuzumab indicated some separation of species with different drug-to-antibody ratios (DARs). According to mass spectrometric analysis, the authors observed that lower DAR species eluted first and higher DAR species later when a shallow gradient was used. Since the attached drug (maytansinoid) is hydrophobic, the hydrophobicity of trastuzumab emtansine increases with the number of attached drug molecules resulting in the observed elution order. Determining the average DAR is a critical quality attribute (CQA) for ADCs and elucidating the distribution of species with different DARs is important to ensure consistent quality and notably efficacy of the conjugates. However, for LC-MS quantitation and DAR distribution studies, chromatographic separation of different DAR species was not sufficient, so the authors had to rely on the resolving power of HRMS and the spectral deconvolution software to distinguish different DAR species.

Size exclusion chromatography (SEC), ion exchange chromatography (IXC), hydrophobic interaction chromatography (HIC) as well as hydrophilic interaction chromatography (HILIC) are all used in the quality control of therapeutic proteins during manufacturing. It would go beyond the scope of this review to discuss each of them in detail, so we will only present a few examples. The reader is referred to the cited literature for further details.

Size exclusion chromatography (SEC) is particularly suitable to address the question of protein aggregation during manufacturing and storage [81,82]. While there have been reports that SEC can be coupled to MS, this is not routine because, unless non-volatile buffer components are used, SEC is not compatible with online coupling to MS. It is, however, possible to collect fractions, desalt the proteins and analyze them by MS. Both anion- and cation-exchange chromatography (IEX) are widely used for the purification as well as for the analysis of proteins. IEX is particularly suitable for charge-variants, notably due to deamidation [83,84] and has been coupled online to MS for the characterization of therapeutic proteins [85]. Hydrophilic interaction chromatography (HILIC) operates on the basis of hydrophilic interactions between the analytes and the stationary phase, which is more hydrophilic than the mobile phase. An inverse gradient of organic solvent is used for elution, which makes HILIC highly complementary to RPLC. Recent work by Somsen et al. [71] showed that HILIC can be coupled online to MS and that different glycoforms of intact proteins can be efficiently separated in this way. Hydrophobic interaction chromatography (HIC) is primarily used as a preparative chromatographic method to purify proteins under native conditions [86]. However, HIC in conjunction with volatile buffer and salt components has recently also been coupled to MS [87]. Whether this will develop into a widely used, robust approach remains to be seen.

The field of high-resolution protein separation by chromatography has not advanced as much as that of high-resolution mass spectrometry, since its inception in the 1980s. This has greatly hampered our current understanding of protein heterogeneity and its implications for protein function. While the challenges are immense and possibly bigger than in the field of MS, it is good to see that the growing field of biopharmaceuticals has revived this area of research and that fundamentally new approaches, such as nanoengineered chromatographic columns, are being developed to advance the field [88].

4. Protein mass spectrometry

Mass spectrometers, especially the widely used triple quadrupole instruments, initially focused on the analysis of small molecules. More recent developments and advances in the field of HRMS have opened new possibilities in the field of protein analysis. Most commonly used HRMS-systems are based on quadrupole time-of-flight (QTOF) and Orbitrap mass analyzers combined with ‘soft ionization’ modes like electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). While ionizing large macromolecules is a challenge in itself, it is further difficult to steer heavy ions through the mass analyzers and the ion optics to the detector resulting in low ion transmission when compared to small molecules and peptides. Advances in hardware and operating conditions (e.g. residual pressures in different sections of the instrument) have improved the capability of QTOF and Orbitrap MS systems to handle higher masses enabling the analysis of

intact antibodies and even viruses. The coupling of ion mobility with mass spectrometry has allowed the separation of ions on the basis of charge and shape inside the mass spectrometer adding another dimension to HRMS systems.

ESI and MALDI can generate intact gas-phase molecular ions from hundreds to millions of Daltons. MALDI relies on ion generation upon irradiation of a light-absorbing matrix in which the proteins are embedded. While the mechanism for ion formation is still not fully understood, the process entails evaporation and activation of the matrix by a laser, generating a plume of hot gas containing ionized matrix ions, which in turn ionize the proteins. MALDI mass spectra of proteins predominantly show singly-charged ions and, to a lesser extent, doubly and triply charged ions. This low degree of charge distribution has the advantage of producing less complicated mass spectra compared to ESI, which makes interpretation easier. MALDI is, however, prone to ion suppression and cannot be easily coupled to LC. It is also difficult to combine MALDI of high-molecular weight molecules with commonly used mass analyzers like ion traps, triple quadrupoles or orbitraps, since ions of increasing mass carrying only a single charge are very difficult to trap efficiently in the quadrupole or orbitrap field regions.

ESI is based on an electrical potential that is applied to the ESI-needle in the source of the MS. This causes a build-up of positive or negative ions, depending on the ionization mode (positive or negative), at the tip of the needle [89–91] leading to the production of charged droplets carrying the proteins. While the droplets evaporate under elevated temperature, the ions inside the decreasing volume of the droplet cause electrostatic repulsion leading to droplet fission, ultimately yielding gas-phase ions. ESI produces mass spectra with multiple charge states, varying from a few to tens of charges per ion, giving a so-called charge state envelope. The width of the envelope is influenced by the state (unfolded/partially folded or folded) of the protein in the liquid phase as well as by the energy the ions receive in the interface of the ESI source. Folded proteins are more compact and the interior amino acids are less prone to be charged than amino acids at the surface of the protein. Thus unfolded proteins tend to have broader charge state envelopes with a higher average number of charges [92]. A wide envelope has the disadvantage that the intensity of the signal is dispersed over many charge states, leading to a loss of sensitivity. In addition, each molecular ion in the envelope is divided into species with a different mass due to the occurrence of natural heavy isotopes such as ^{13}C, the so-called isotopologues. The signal for large proteins with many isotopologues is thus spread out over even more ions, leading to a further loss in sensitivity. With increasing number of charges, the isotopologue peaks within one charge state will be spaced ever more closely ultimately merging into a single peak whose width is determined by the isotopologue cluster. The charge state envelope can be modified by adding chemicals [93], so-called superchargers, to the LC-flow (e.g. post-column) [94] or to the gas-phase of the ESI source [95]. While these chemicals were first used in the field of peptide analysis [96], they have also been applied for intact protein analysis [93–95,97,98]. Super-charging shifts the charge state envelope to higher charge values, thus lowering the m/z ratios. This improves ion transmission and brings the m/z values within the range of most commonly used mass analyzers (notably quadrupoles). Reducing the width of the charge state envelope may also increase sensitivity by collecting more ions per charge state resulting in an increase in peak intensity. Fig. 3 shows the shift in the charge state envelope of recombinant human growth hormone (rhGH) upon addition of metanitrobenzylalcohol (NBA), a supercharging agent, to the mobile phase after the chromatographic separation. In the lower trace (magenta) of Fig. 3 it is noticeable that the sensitivity of rhGH is increased besides shifts in the charge state envelope. However, this is also the case for the background and signals arising from possible co-eluting adducts ($Na+$ and $K+$). There should thus be a thorough investigation of the effect of supercharging on the S/N ratio, especially near the lower limit of quantification.

The field of super-charging is still in its infancy and it remains to be seen whether other additives or other means of doping the electrospray to increase the average charge state and to reduce the width of the charge state envelope will lead to more sensitive and possibly more selective detection of intact proteins upon LC-MS. An attractive option was recently shown by Somsen et al. [71], who doped the gas phase in the ion source rather than the mobile phase of the LC separation. While the main goal of these authors was to reduce the ionization suppressive effect of TFA, they also observed that adding 1% propanoic acid to the drying gas increased the charge state and reduced the number of TFA adducts. This approach merits further study, since it allows to affect the charge state envelope without interfering with the chromatographic separation.

Another way of reducing the width of the charge state envelope is to use so-called 'native mass spectrometry', meaning mass spectrometry under conditions that do not denature proteins or that denature them only partially [99–101]. As the protein maintains (part of) its 3-dimensional structure, it acquires fewer charges through protonation or the association with other cations. This facilitates the resolution of proteoforms in the m/z domain in contrast to supercharging, which puts more demands on the resolution of the mass analyzer. However, native mass spectrometry places considerable constraints on the mobile phase conditions for the LC separation, the instrumental set-up and the conditions for effective ion transmission (e.g. potentials and residual pressures). LC-MS under 'native' conditions is thus currently not as sensitive as LC-MS under denaturing conditions. Considering that efficient protein separation by LC requires elevated temperatures, complicates the application of native MS to the bioanalysis of proteins and notably protein species. Native mass spectrometry is thus mainly used for product characterization when a large amount of purified therapeutic protein is available for analysis [43,102–107]. Since most proteins (e.g. mAbs) acquire their specificity due to a well-defined 3-dimensional structure [108], it is conceivable that native LC-MS may also be used to distinguish biologically active forms of a protein from inactive forms (e.g. when combined with the results of activity assays). This makes native LC-MS a subject that is worth exploring further for the bioanalysis of protein species and more generally to investigate protein-protein and protein-small molecule interactions.

Recently the use of ion mobility spectrometry (IMS) has entered the field of protein analysis [104,106,109–111]. ~~An IMS cell is generally placed between the ion source and the first quadrupole.~~ IMS may be described as 'gas-phase electrophoresis' and introduces an extra separation dimension after the LC separation and ahead of the actual mass analyzer. Separation is based on the physical characteristics of proteins such as lipophilicity, shape and charge [112]. The mobility of ions along the IMS cell is based on the number of interactions with the neutral gas under the influence of a potential gradient, which slows the ions down. Within a single charge state, folded proteins drift faster than unfolded proteins because they experience fewer interactions with the buffer gas, and thus have a smaller collisional cross-section. The most widely used types of IMS cells are drift tubes, the field asymmetric wave ion mobility cell [113] and the traveling wave IMS (T-Wave). In the first technique, the proteins move in the drift tube under the influence of a constant electrical field, while the second technique uses electric fields modulated by radio frequencies that are produced by coils. T-Wave IMS utilizes non-uniform, moving electric field/voltage pulses to push ions through a neutral buffer gas. Species with high mobility (lower collisional cross-section) surf more on the wave front and are overtaken by the wave less often than species of low mobility (higher collisional cross-section). At present, IMS is predominantly used for biopharmaceutical product characterization and to evaluate disulfide bond heterogeneity, conjugation heterogeneity (e.g. in ADCs), analysis of protein aggregation and global conformational changes [114–120]. It must be noted that gas phase conformations (evaluated by IMS) may be related, but are not identical to solution-phase conformations. A topic that requires further investigation and which is of utmost interest for

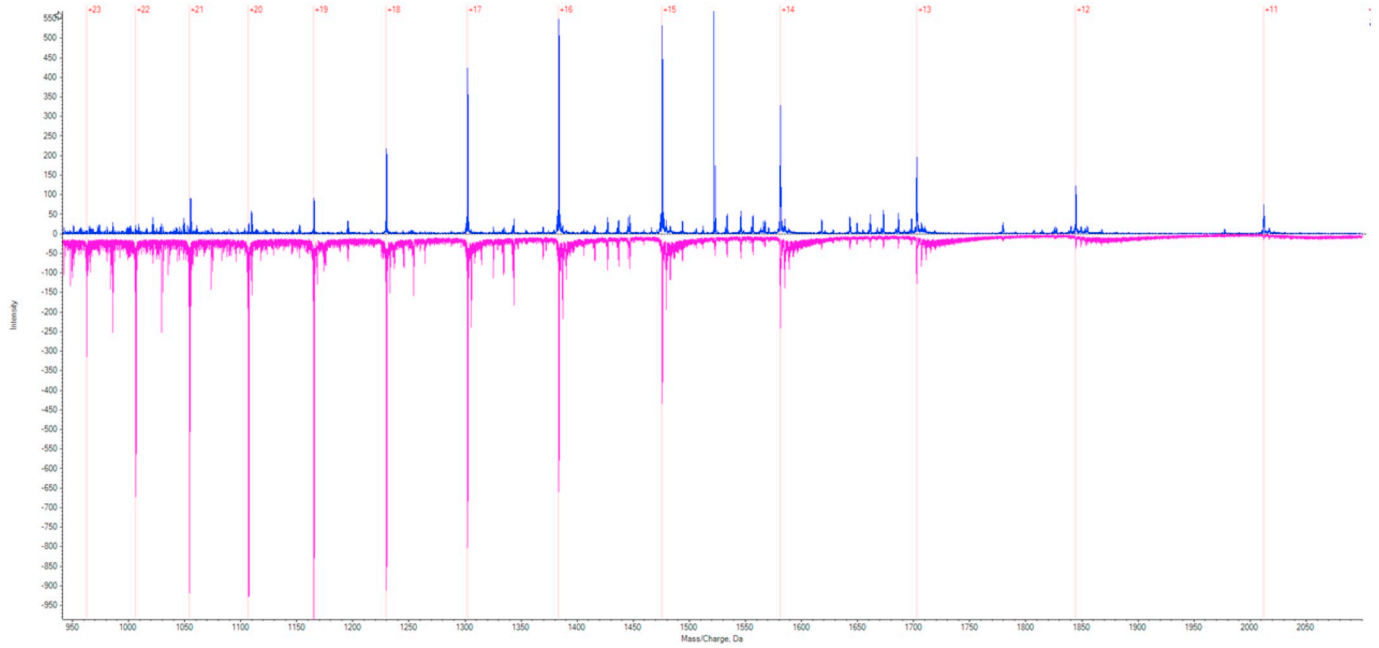

Fig. 3. Shift of the charge state envelope for recombinant human growth hormone (rhGH) to higher z values upon addition of metanitrobenzylalcohol (NBA) to the mobile phase after the chromatographic separation. Upper trace (blue); no addition of NBA; Lower trace (magenta); with post-column addition of 0.16% (v/v) of NBA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the field of 'native' mass spectrometry. Another caveat of IMS is currently that it decreases ion transmission and thus sensitivity considerably, which may be one reason why it is not commonly used.

5. Processing and analysis of intact protein LC-MS data

LC-MS analyses of intact proteins produce complex raw data consisting of retention times, peak heights or areas and *m/z* ratios. Since most proteins do not exist as single molecular entities, each protein species gives rise to a slightly different charge state envelope. The fact that many protein species are not chromatographically resolved results in overlapping charge state envelopes, each with their isotopologue distribution. To complicate matters further, proteins often form non-covalent adducts with cations such as Na^+ or K^+, which adds to the complexity of the resulting ESI-MS spectra. Together, this makes conversion of the information provided in the ESI-MS raw data into simple readouts, such as the amount or concentration of a given protein species or its molecular mass, rather difficult. Examples of the mass spectrometric output for proteins of increasing molecular mass are shown in Fig. 4.

For a relatively small protein such as IGF-1 (molecular mass of 7.5 kDa, Fig. 4A), the number of charge states is limited to just a few. As mass increases, proteins acquire more and more charges (Fig. 4B and C) so that for a complete mAb such as trastuzumab (mass of around 145 kDa, Fig. 4D), > 40 different charge states can be formed during ESI, with the number of charges varying from around 30 to over 70. Fig. 4 also shows that for smaller proteins the isotopologues of a given charge state can be resolved in a TOF mass analyzer with a resolution of 30,000. For proteins with masses of > 25 kDa, the resolution of this mass analyzer is insufficient to resolve the isotopologues and a mass spectrometer with a considerably higher resolution would be needed for a complete separation of their signals.

Typically, for each data point in a chromatogram a full mass spectrum is acquired, containing the response of all ions corresponding to the various charge states of a protein that elutes at the particular retention time. An EIC is then constructed by defining a small MEW around the *m/z* value that corresponds to the ion of interest. As an example, Fig. 5 shows an EIC of the 20^+ charge state of the Fc/2 fragment and the 44^+ charge state of the $F(ab')_2$ of 1 μg/mL of trastuzumab, after immunoaffinity enrichment from human plasma and IdeS digestion.

Increasing the width of the MEW may be beneficial, as a larger number of analyte ions will be detected, and the sensitivity therefore improved. At the same time, it may also result in the co-extraction of ions from interfering matrix proteins that elute at the same time and have an *m/z* value close to the protein of interest [121]. Alternatively, it is possible to select only one of the most abundant charge state ions for quantification and to optimize the width of the EIC extraction window in order to reach an optimal S/N ratio. Ruan et al. [66] showed that by selecting the most abundant charge state of lysozyme, it is possible to improve the S/N ratio from 12 to 40 upon reducing the MEW from 0.5 *m/z* to 10 ppm.

Fig. 6 shows the effect of decreasing the MEW from 1.0 Da (entire 15^+ charge state) to 0.25 Da and ultimately to 0.0625 Da (1 isotope of the 15^+ charge state isotope cluster) for the 15^+ charge state of recombinant human growth hormone (rhGH) after enrichment from rat plasma. The results show that decreasing the EIC extraction window does not lead to significant improvements in S/N ratio in this case and while the absolute signal intensity decreases, the selectivity increases due to less interference. It has to be noted, that adjusting the EIC extraction window may improve the S/N ratio in other cases depending on the nature of the background.

Simplifying protein ESI-MS spectra based on the deconvolution of each charge state envelope into a single peak giving the average molecular mass of the protein (or protein species) is an attractive approach. The mathematical process of converting the measured *m/z* values into molecular masses starts with assigning a charge state to a given ion. This can be achieved based on the knowledge (assumption) that two neighbouring peaks from the same protein molecule are separated by a single charge and that this charge is due to the attachment of a hydrogen cation (also called a 'proton' or simply an H^+ ion). If this assumption is correct, one can use simple algebra to define the charge state of a given protein ion in a charge state envelope (see Box 1).

To calculate molecular masses from charge state envelopes, algorithms based on different principles have been developed. The so-called 'Maximum Entropy (MaxEnt)'-based approach, which forms the basis of

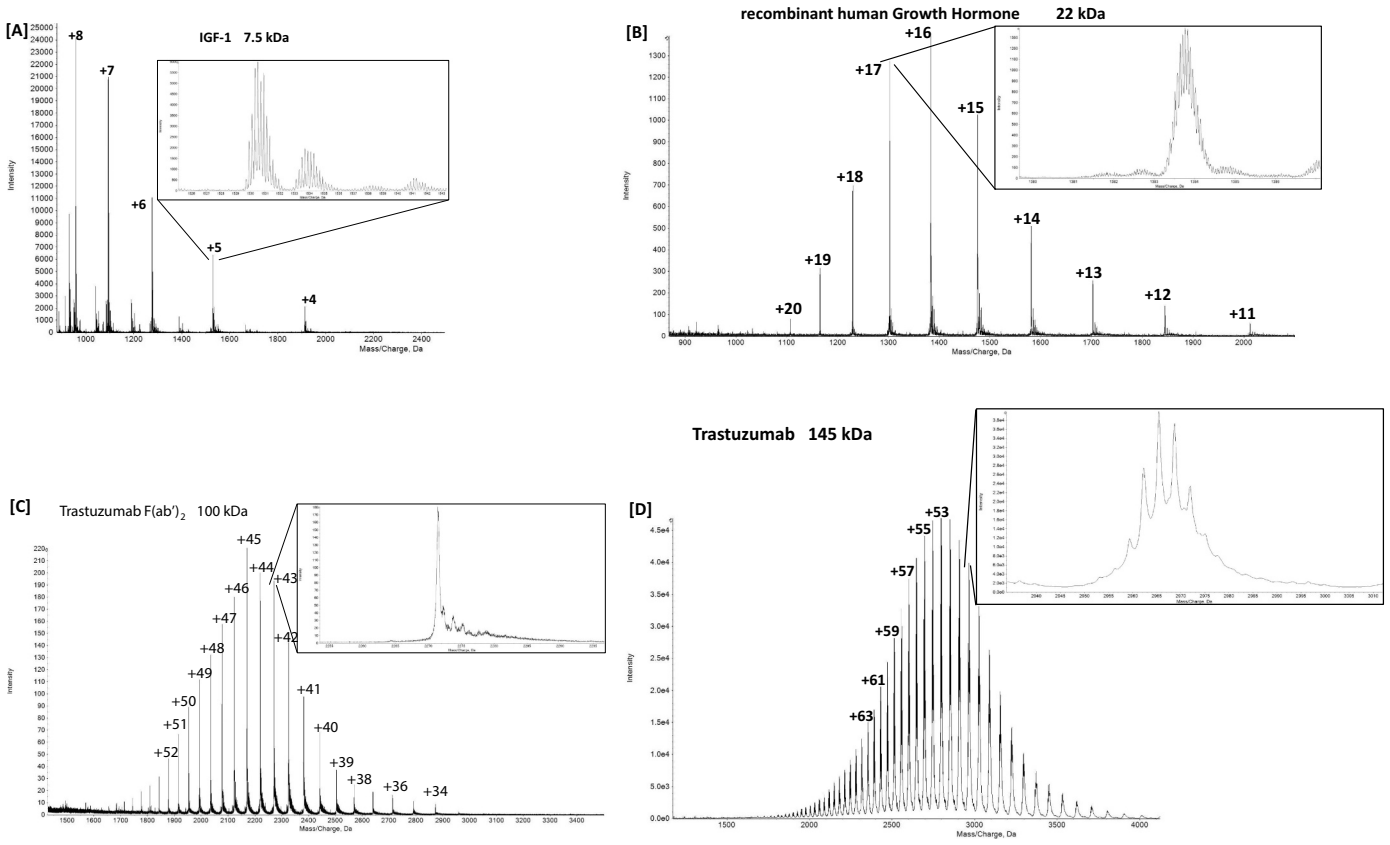

Fig. 4. Charge state envelope of IGF-1 (A), recombinant human growth hormone (B), the F(ab′)$_2$ part of trastuzumab (C) and trastuzumab (D) with a zoom into one charge state, as indicated in the figure (data acquired with a QTOF mass spectrometer at a resolution of approx. 35,000 at m/z 900). While the isotopologue cluster of IGF-1 is still resolved, this is no longer the case for the larger proteins. The zoom spectra indicate the presence of different protein species.

most commercially available software packages, tries to take peak broadening effects, due to unresolved isotopes and limited resolution of the mass analyzer, into account thereby improving the original quality of the spectrum (higher resolution and reduction in noise). The MaxEnt program, as developed by Skilling et al. [122], processes different in silico generated trial spectra and compares them to the observed spectrum. Once a predefined level of convergence has been reached, trial spectra that agree with the original data compose the final probability distribution of spectra in *m/z* scale. The spectrum with the highest entropy, the MaxEnt spectrum, is chosen as the most probable representation of the original data. The width of the probability distribution reflects the uncertainty of the MaxEnt result, which is

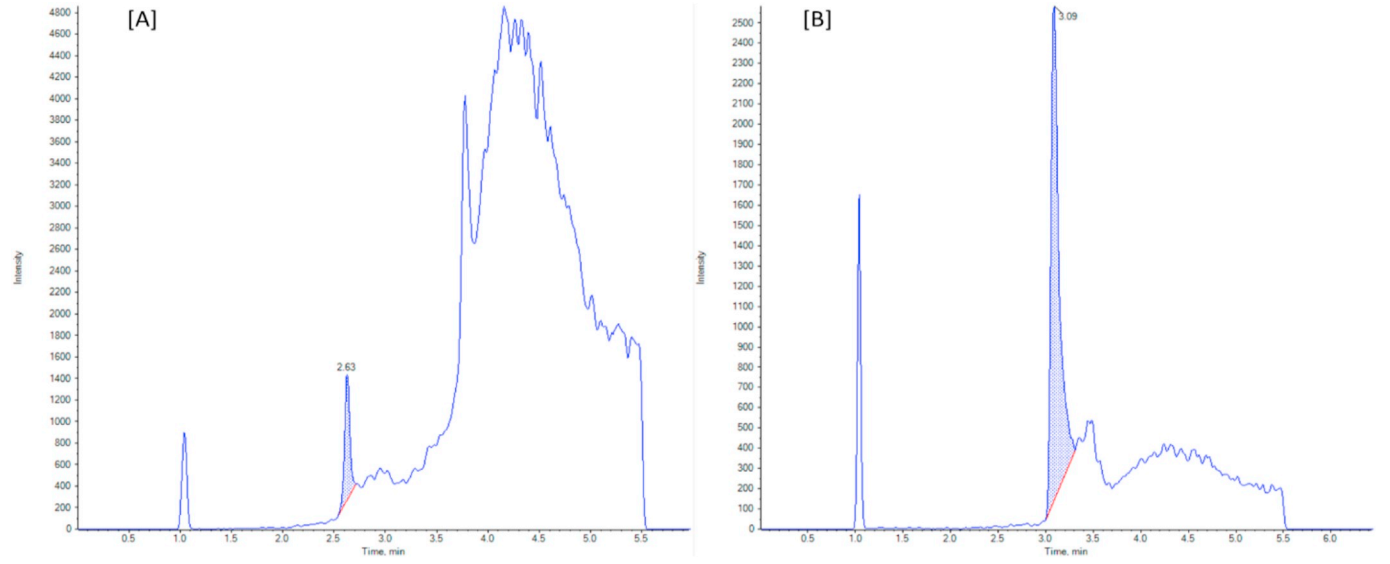

Fig. 5. LC-MS chromatograms (EICs) of 1 µg/mL trastuzumab in rat plasma, after immunoaffinity enrichment using the HER2-receptor as capturing agent and IdeS digestion. (A) the 20$^+$ charge state of the Fc/2 fragment (2.63 min) and (B) the 44$^+$ charge state of the F(ab′)$_2$ fragment (3.09 min). Separation was by reversed-phase LC on a C$_4$ column at 80 °C and detection on a QTOF mass spectrometer at resolution 30,000. The MEW used was 0.5 *m/z* units.

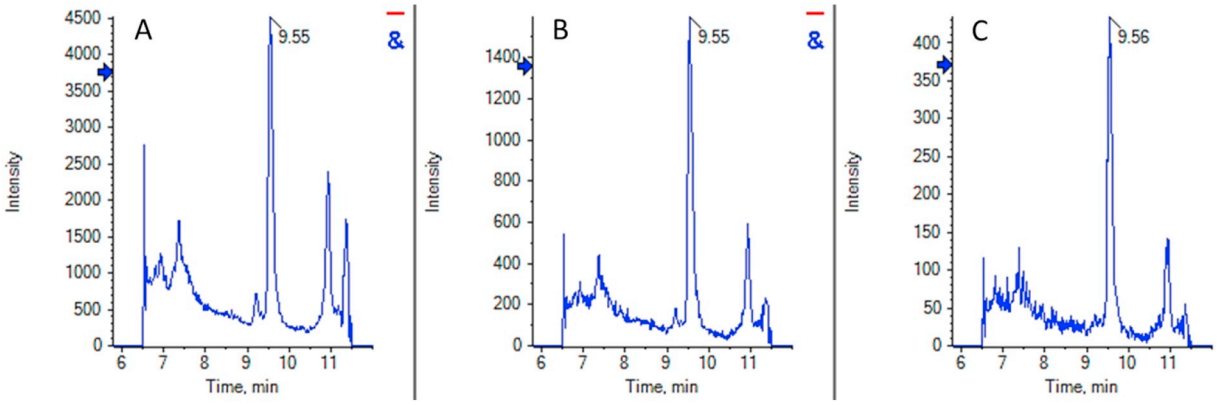

Fig. 6. LC-MS chromatograms (HRMS) of 10.0 ng/mL of recombinant human growth hormone after immunoaffinity enrichment from rat plasma. EICs for the 15^+ charge state were recorded with an MEW of 1.0 Da (A), 0.25 Da (B) or 0.0625 Da (C).

presented in form of error bars. Since peaks in an ESI-MS charge state envelope are intrinsically connected (see Box 1), this criterion is used to qualify the MaxEnt spectrum, as it must explain the entire charge state envelope above a pre-defined threshold across the selected *m/z* range. However, the MaxEnt algorithm does not need to assign a specific charge state to a given peak to start with. It rather uses all of the information to connect 'peaks that belong together'. It is noteworthy to mention that considerable improvements in mass accuracy and resolution of modern mass analyzers facilitate accurate deconvolution of ESI-MS spectra even for large biopharmaceuticals, such as monoclonal antibodies [70]. However, they do not take all of the caveats away. That is why further developments in both mass spectrometry (e.g. reducing the number of charges per protein ion by 'native' mass spectrometry) and algorithms are ongoing [99].

Fig. 7 shows the results of deconvoluting the charge state envelope of the Fc/2 subunit of trastuzumab using a maximum-entropy-based algorithm. While the deconvoluted mass (25,231.6) of the left major peak corresponds closely to the expected mass of 25,232.0 Da of one glycoform of trastuzumab (Fc/2 G0F) with the loss of a Lys residue from the C-terminus, there are other smaller signals that indicate additional protein species or non-covalent adducts (see insert). It is currently still under investigation whether this kind of transformation results in valid quantitative data [70].

While deconvolution makes subsequent data analysis and

Box 1

Example of a calculation to derive the molecular mass of the Fc/2 fragment of trastuzumab (after IdeS digestion).

Figs. 4 and 7 shows the ESI spectrum of the Fc/2 part of trastuzumab. To determine the charge state of the ion at 1262.5632 (P_1) of the charge state envelope of the Fc/2 part of trastuzumab, we consider that the following peak at 1328.9577 (P_2) has one H^+ less and that $z_1 - z_2 = 1$. The mass difference between the two protein ions is thus 1.00794 amu (atomic mass units), the mass of an H^+ ion. This knowledge allows us to calculate the charge state of P_1 and from thereon to derive all other charge states based on the assumption that each consecutive peak towards increasing *m/z* values in the spectrum is due to the loss of one H^+ ion.

From the measured data we derive:

$P_1 = m/z_1 = 1262.5632$ and $P_2 = m/z_2 = 1328.9577$

From the assumption that each charge state is due to a difference in one H^+ ion, we derive:

$z_1 - z_2 = 1$ or $z_1 = z_2 + 1$

Thus: $m = M - z \cdot H^+$; with m being the molecular mass of the protein and M the molecular mass of the respective molecular ion of charge state z.

Referring back to the measured values P_1 and P_2 results in:

$P_1 = M - z_1 \cdot H^+ / z_1$ or $P_1 = M - (z_2 + 1) \cdot H^+ / z_2 + 1$ and $P_2 = M - z_2 \cdot H^+ / z_2$

Resolving these equations with respect to M gives:

$P_1 \cdot z_1 + z_1 \cdot H^+ = M$ and $P_2 \cdot z_2 + z_2 \cdot H^+ = M$

Which can be rearranged to:

$P_1 \cdot z_1 + z_1 \cdot H^+ = P_2 \cdot z_2 + z_2 \cdot H^+$ or $P_1 \cdot z_1 + z_1 \cdot H^+ = P_2 \cdot (z_1 - 1) + (z_1 - 1) \cdot H^+$

Resolving the equation on the right with respect to z_1 gives:

$z_1 = -(H^+ + P_2) / P_1 - P_2$

This allows to calculate the number of charges z_1 based on the measured values of P_1 and P_2.

Inserting the measured values and using the known mass of the H^+ ion of 1.00794 gives:

$z_1 = -(1.00794 + 1328.9577) / 1262.5632–1328.9577 = 20.03$, which is close to $z_1 = 20$, as charge states can only acquire integer values.

With $\mathbf{z_1 = 20}$, we can now calculate the approximate molecular mass of the Fc/2 fragment of trastuzumab as:

$M + 20 \cdot H^+ / 20 = 1262.5632$ or $M = 1262.5632 \cdot 20–20$ or $\mathbf{M = 25{,}231.26\ Da}$

The same calculation for $z_2 = 19$ gives:

$M + 19 \cdot H^+ / 19 = 1328.9577$ or $M = 1328.9577 \cdot 19–19$ or $\mathbf{M = 25{,}231.20\ Da}$

Both values are quite consistent and correspond closely to the expected mass of 25,232.0 Da. By going through the entire charge state envelope in this manner (using a computer algorithm), we get a list of calculated molecular masses that differ slightly from each other giving an average value with an estimate of the measurement error.

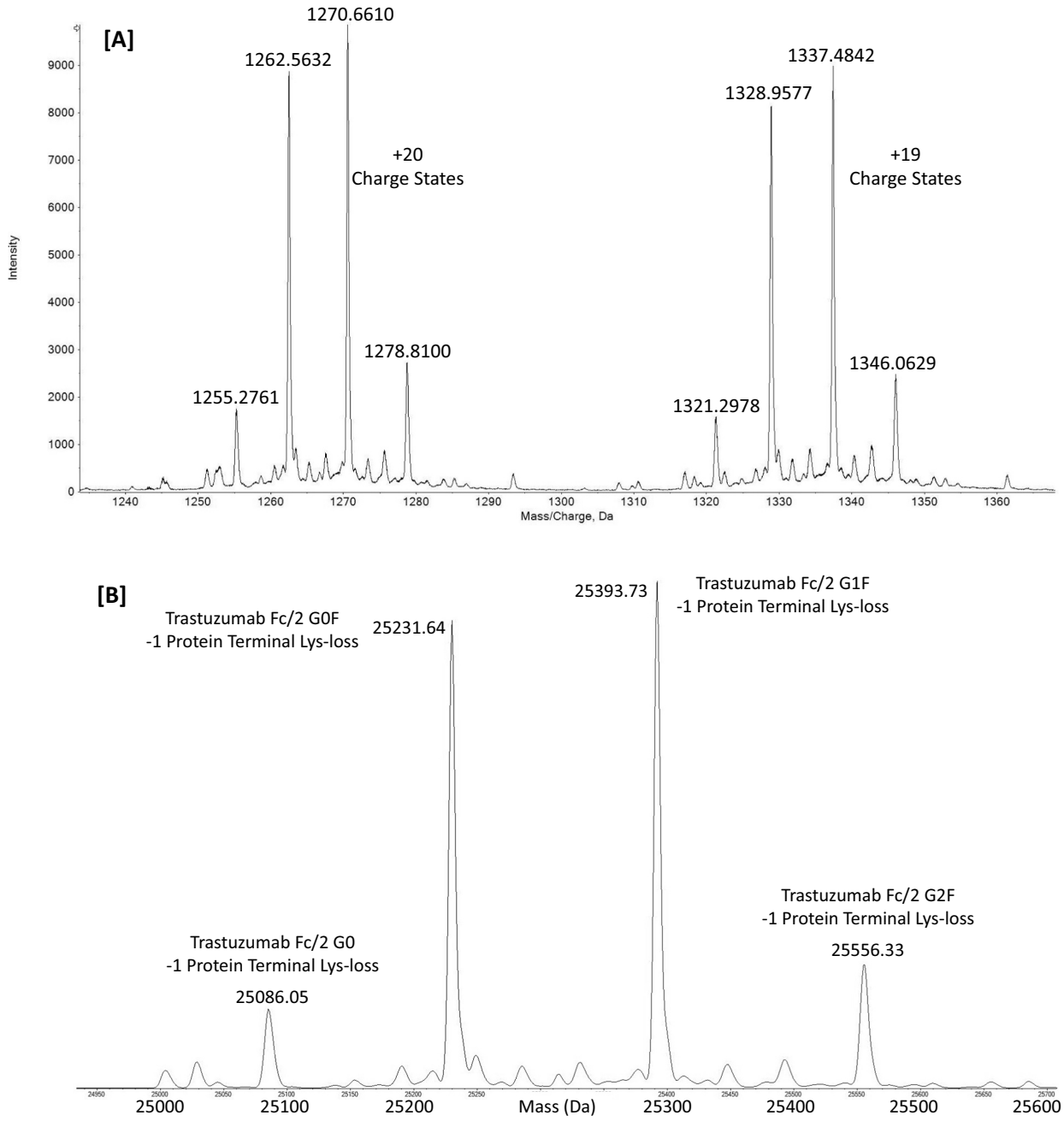

Fig. 7. Deconvolution of the charge states 20^+ and 19^+ of the Fc/2 fragments [A] of trastuzumab after IdeS digestion using a maximum entropy-based algorithm [B] integrated into proprietary software (SCIEX).

quantification considerably easier, it is prone to artefacts. Mathematical deconvolution algorithms may introduce bias and errors and may not give an accurate reflection of the intensity of the original signals. The result of such a deconvolution process depends further on the chosen parameters such as peak width, signal threshold and the chosen *m/z* range. Deconvolution of charge state envelopes from ESI-MS spectra of different protein species requires their chromatographic separation or, if this cannot be achieved, sufficient resolution of the mass analyzer to resolve the charge state envelopes in order for the algorithm to assign different deconvoluted signals to each form. The occurrence of non-covalent adducts (e.g. Na^+ or K^+), which is quite common in protein ESI-MS spectra, as well as modifications resulting in small mass differences (e.g. due to deamidations) may confound the deconvolution algorithm leading to artefacts with incorrect mass and peak heights or areas. The use of deconvolution algorithms in regulated, quantitative bioanalysis thus represents a considerable challenge.

6. Conclusions and future outlook

The analysis of intact proteins by LC-MS receives considerable interest from industry and academia alike. After initial breakthroughs in the 1980s due to improved chromatography materials (e.g. wide-pore silica beads) and due to a better understanding of the chromatographic separation of macromolecules from a theoretical point of view, there were many years of little progress in this field. The emergence of recombinant proteins as therapeutic drugs and their increasing relevance from a medical as well as from a commercial point of view, has created renewed interest and led to improvements of protein separations by LC (e.g. based on core-shell particles and monoliths) in conjunction with tailor-made surface chemistries in combination with high-resolution

mass spectrometry. Ongoing development of HRMS-systems and new affinity agents will aid in studying in vivo protein biotransformation in greater detail possibly leading to a better understanding of how this affects therapeutic efficacy.

Efficient separation of proteins by (U)HPLC has turned out to be a much more difficult task than initially anticipated. The low diffusion coefficient of proteins results in slow mass transfer kinetics and the possibility to assume multiple interchanging conformations has rendered efficient chromatographic separations more challenging than for peptides. New developments in the field, for example, the emergence of columns containing nanoengineered micropillars, hold promise of improving analytical protein separations, but we must remember that the fundamental physical properties of proteins cannot be changed. They will define the limits of protein separations by liquid chromatography.

In this review, we have given an overview of the area of intact protein (bio)analysis by LC-MS starting with the challenges of sample preparation and ending with the deconvolution of highly complex mass spectra after electrospray ionization. We notably focused on the LC-MS analysis of intact proteins in biological matrices, an area that is in its infancy but that is instrumental for the further development of therapeutic proteins, for the detailed characterization of biomarkers and for biological and biomedical research in general. While there is still a long way to go until LC can rival two-dimensional gel electrophoresis with respect to intact protein separation, the payback for advances in this area will be large, since protein heterogeneity, for example due to post-translational modifications, is inherent to the way how nature modulates the biological activity of proteins and of biological systems in general. We will not be able to understand this in sufficient detail unless we develop the necessary bioanalytical tools.

Acknowledgements

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